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Application of:	Segal	Examiner:	Belyavskyi, M
Serial No.:	09/318,870		
Filed:	May 26, 1999	Group Art Unit:	1644
Entitled:	Cytokine Coated Cells and Methods of Modulating an Immune Response to an Antigen	Conf. No.:	2018

Mail Stop AF
Commissioner for Patents and Trademarks
Washington, D.C. 20231

RULE 132 DECLARATION OF DR. ANDREW SEGAL

I, Andrew Segal, hereby declare that:

1. I am the sole inventor of the above referenced patent application. I received a B.A. in Biochemical Sciences from Harvard University in 1985, and an M.D. from Boston University School of Medicine in 1990. I conducted postdoctoral research from 1994 to 1996 at the Whitehead Institute of Biomedical Research. I am currently the President and CEO of Genitrix, LLC.

2. I have read the Office Action mailed in the above-referenced patent application on April 20, 2003, and understand that questions have been raised by the Examiner as to whether the disclosure provided by the specification is sufficient to support the breadth of the claimed invention.

3. The experiments described herein were performed under my direction. These experiments were performed *in vivo* in mice. The data described herein show that an engineered cytokine comprising the cytokine GM-CSF fused to a heterologous cell membrane binding moiety is mixed with fibrosarcoma cells, B16F10 murine melanoma cells, or K1735 melanoma cells to produce a vaccine, prior to the administration of the vaccine to a mammal. The results demonstrate that the administration of these vaccine compositions in an amount within the ranges taught in the specification, to a mouse inhibits tumor growth compared to the administration of a control vaccine which does not comprise a cytokine coated cell. The

experiments described herein were performed according to the teachings of the above captioned patent application, and thus support the operability of the methods and vaccine compositions described therein.

The data described herein shows that three different vaccine compositions are demonstrated to be effective in vaccinating a mammal as claimed in the present invention. As described below, cytokine coated CMS-5 fibrosarcoma cells are used to vaccinate mice against subsequent CMS-5 tumor cell challenge. In these experiments, GM-CSF-K-HA refers to a fusion protein comprising the cytokine GM-CSF fused via a linker (K) to a heterologous membrane binding domain, influenza hemagglutinin (HA). The methods used to construct the GM-CSF-K-HA have also been provided for completeness. In addition, experiments are described below in which vaccination of a mouse against challenge with B16F10 melanoma cells by administering to the animal a vaccine composition comprising B16F10 melanoma cells mixed with GM-CSF-K-HA. The experiments below also describe the vaccination of mice using a vaccine composition comprising K1735 melanoma cells mixed with GM-CSF-K-HA, and vaccination using a vaccine composition comprising CT26 colon carcinoma cells mixed with GM-CSF-K-HA. The experiments described herein were performed as described in detail in the above-referenced patent application.

Cloning of GM-CSF/Influenza Hemagglutinin Chimeric Proteins

pUC19 GMCSF-K-GAS1.1

pUC19 GM-CSF-K-HA was cloned starting with pUC19 GM-CSF-K-Gas1.1, which we produced in our laboratory. This plasmid includes a sequence that encodes murine GM-CSF fused to a downstream glycosylphosphatidylinositol modification sequence derived from the yeast GAS1 protein (the latter obtained from Dr. D. Wittrup, University of Illinois). A linker sequence is interposed between the GM-CSF and GAS1 portions. To insert the linker sequence, the plasmid pUC19 GMCSF-Gas1.1, also previously produced in our lab, was digested with NgoM IV and NheI. These restriction enzymes cut at the 3' end of the GM-CSF molecule and at the 5' end of the Gas1.1 sequence, respectively. The resulting plasmid was purified after electrophoresis through agarose gel using a kit manufactured by Qiagen. The following oligonucleotides were purchased :

5' CCGGCACTAGTGGCGGAGGGGGCTCCGGCGGCGGGGGCAGCG

5' CTAGCGCTGCCCCCGCCGCCGGCGCCCCCTCCGCCACTAGTG

The synthetic oligonucleotides contain:

1. 5' overhang that anneals to NgoM IV digested plasmid DNA

2. 3' overhang that anneals to Nhe I digested plasmid DNA

3. DNA sequence coding for the peptide GGGGSGGGGS where G stands for glycine and S stands for serine. This 10 amino acid sequence $(G_4S)_2$ is designed to insert a kink/spacer in the protein between the GMCSF and the Gas1.1 moieties.

4. SpeI site to allow confirmation of cloning of the small fragment and for further manipulations.

The two oligonucleotides were mixed in equimolar concentrations, boiled for 2 minutes and allowed to anneal at room temperature. The oligonucelotide was ligated into the NgoM IV - Nhe I digested plasmid and the plasmid was used to transform the E.coli strain AG-1. Transformants were selected on LB plates containing 100ug/ml ampicillin. Plasmid DNA was isolated, digested with Spe I, and electrophoresed on agarose gels to confirm the presence of the $(G_4S)_2$ sequence.

pUC19 GM-CSF-K-hemagglutinin (HA)

The plasmid pUC19 GM-CSF-K-HA was produced, which encodes a chimeric protein containing (from amino terminal to carboxy terminal): (1) murine GM-CSF; (2) the $(G_4S)_2$ linker described above; and (3) the HA1 domain of the H1 HA from the A/PR/8/34 influenza A isolate. The HA1 sequence used (amino acids 18 to 344 of the HA precursor) omits the N-terminal leader sequence and the downstream HA2 domain. A termination codon was added after amino acid 344.

pUC19 GM-CSF-K-Gas1.1. was digested with Nhe I and Kpn I. Nhe I cuts at the 5' end of the Gas1.1 coding sequence and Kpn I cuts at the 3' end of the Gas1.1 coding sequence,

respectively. The resulting plasmid with the GPI coding region removed, was purified after electrophoresis through agarose gel using a kit manufactured by Qiagen. The HA1 coding sequence was cloned by PCR from a plasmid encoding the HA gene of the A/PR/8/34 strain of influenza. The HA1 sequence used begins at amino acid 18, the start of the mature protein, i.e. lacking the secretion signal sequence. The 3' end corresponds to amino acid 344, eliminating the transmembrane region and substituting a termination codon. Primers for PCR of the HA1 sequence were as follows:

Upstream HA1 Primer

5' ATGCTAGCGACACAATATGTATAGGC

Downstream HA1 Primer

5' ATGGTACCCGGCCGTTATCATCTGGATTGAATGGACGG

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute
	Extension	72^0	one minute

PCR was performed for 20 cycles using vent polymerase.

Following PCR, the product was electrophoresed through a 1.0% agarose gel and the HA1 cDNA was extracted from the gel using a Qiagen kit according to the manufacturer's instructions. The purified HA1 DNA fragment was digested with Nhe I and Kpn I. To make the fusion protein, the purified Nhe I – Kpn I HA fragment was ligated into the pUC 19 GM-CSF – K- Gas1.1 vector that had been digested with Nhe I and Kpn I to remove the Gas1.1 coding region. The DNA was used to transform E.coli AG1 and transformants selected on LB-ampicillin plates. Plasmid DNA from individual colonies was isolated and digested with restriction enzymes. Restriction digests identified a pUC19 GM-CSF-K-HA plasmid.

The pUC19 GM-CSF-K-HA plasmid was purified according to the manufacturer's instructions using a kit purchased from Qiagen.

Cloning of GM-CSF-K-HA into Yeast Expression Vector

PCR of pUC19 GM-CSF-K-HA was used to isolate a DNA fragment encoding GM-CSF-K-HA for cloning into a yeast expression vector. The PCR product contains Eag I cloning sites for in frame insertion into the yeast expression vector.

Upstream Primer

5' TACGGCCGGCACCCACCCGCTCACCC

Downstream Primer

5' ATGGTACCCGGCCGTTATCATCTGGATTGAATGGACGG

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute
	Extension	72^0	one minute

PCR was performed for 20 cycles using vent polymerase.

Following PCR, the product was electrophoresed through a 1.0% agarose gel and the GM-CSF-K-HA gene was extracted from the gel using a Qiagen kit according to the manufacturers instructions. The purified DNA fragment was digested with Eag I and ligated to the yeast expression vector ITK that had been digested with Eag I. The ITK vector is designed for (1) replication in E.coli and (2) expression of genes in the yeast Saccharomyces cerevisiae after stable integration using homologous recombination. The vector contains:

1. Sequences for replication of the plasmid in E.coli

2. Yeast Gal promoter for expression of heterologous genes in yeast grown in media containing galactose.

3. PrePro – Synthetic DNA sequence, optimized for secretion and signal sequence cleavage of distal genes in yeast.

4. Unique Eag I site for cloning genes to be expressed.

5. Alpha terminator- DNA sequence for efficient termination of proximal genes.

6. Delta sequence that allows for stable integration of the plasmid by recombination with endogenous delta sequences in the yeast chromosome.

7. Antibiotic resistance gene allowing for selection in E.coli with kanamycin and selection in yeast with G418.

This plasmid was used to transform E.coli strain AG-1. Transformants were selected by growth on LB plates containing 100 ug/ml kanamycin. Individual colonies were grown in LB media containing kanamycin and plasmids were purified. Restriction digests determined orientation of inserts. The resulting plasmid ITK GM-CSF-K-HA was purified using a kit purchased from Qiagen according to the manufacturer's instructions.

Expression of GM-CSF-K-HA in Yeast

The purified plasmid was linearized with Mfe 1 and used to transform the yeast strain Saccharomyces cerevisiae WDHY131 using lithium acetate (LiAc). A 10 ml culture of S.cerevisiae grown to saturation at 30^0 in YPD media (per liter/ 20g Bactotryptone; 20 g dextrose; 10g yeast extract) was used to inoculate 100 ml of YPD. The culture was grown at 30^0 with shaking for 3 hours. The yeast were harvested by centrifugation at 11,000 x g for 2 minutes and resuspended in 25 ml of sterile water. The yeast were centrifuged as above and resuspended in 1.0 ml of 100mM lithium acetate and transferred to a 1.5 ml microfuge tube. The yeast were pelleted by centrifugation at 12,000 x g for 15 seconds and the supernatant removed. The cells were resuspended in 0.5 ml of 100mM LiAc. 50 uL of cell suspension was added to individual microfuge tubes and centrifuged as above. Supernatant was removed.

Transformation mix added to the yeast pellet consisted of : 240 uL PEG (50% w/v); 36 uL 1.0 M LiAc; 5 uL single stranded DNA (10mg/ml) and 1 ug of linearized ITK GM-CSF-K-HA in 75 uL of water. The mixture was vortexed to resuspend the cell pellet and incubated at 30^0 for 30 minutes. The cells were then shocked at 42^0 for 15 minutes, centrifuged to pellet cells and resuspended in 0.5 ml of YPD. Yeast were incubated in YPD media for 3 hours and plated on YPD plates containing 2 mg/ml G418. Plates were grown at 30^0 for 3 days until individual colonies appeared. To screen for expression of GM-CSF-K-HA, individual colonies were grown

in 1 ml of YPD media at 30^0 for 2 days. The cells were centrifuged at 8,000 x g for 2 minutes and the YPD media removed and replaced with 1 ml of YPG media (per liter/ 20g Bactotryptone; 20g galactose; 10g yeast extract) for induction from the gal promoter. Yeast were grown in YPG media for 2 days. At this time, an aliquot was removed and cells were pelleted. The supernatant was tested for GM-CSF expression using an ELISA kit purchased from Endogen. Protocol was according to the manufacturer. A high-expressing yeast clone secreting the chimeric protein GM-CSF-K-HA was identified. Based on standard curve of soluble GMCSF, expression level was approximately 2.4 mg/L of GM-CSF moiety.

Production of pUC19 HA (hemagglutinin) – K- GM-CSF

The plasmid pUC19 HA-K-GM-CSF was also produced, which encodes a chimeric protein containing (from amino terminal to carboxy terminal): (1) an HA1 domain (2) K, the $(G_4S)_2$ linker described above, and (3) murine GM-CSF, The HA1 begins at the amino terminus of the mature protein, amino acid 18, eliminating the leader sequence. The 3' end terminates at amino acid 344. The $(G_4S)_2$ has been added to supply a flexible linker. The GM-CSF begins at amino acid 18 of the GM-CSF protein, corresponding to the first amino acid of the mature protein.

The HA-K sequence was first cloned by PCR of the HA1 coding sequence from a plasmid encoding the HA gene of the A/PR/8/34 strain of influenza.

Upstream Primer

5' CTGAATTCCGGCCGGACACAATATGTATAGGC

Downstream Primer

5'
ATGGTACCGCTGCCCCCGCCGCCGGAGCCCCCTCCGCCACTTCTGGATTGAATGGAC
GGAAT

The oligonucleotides for PCR generate a nucleic acid with:

1. A 5' EcoRI site at the amino terminus of the mature HA

2. The $(G_4S)_2$ linker at the carboxy terminus of the HA1 domain (amino acid 344 of the HA precursor)

3. A Kpn I site distal to the end of the $(G_4S)_2$ sequence

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute
	Extension	72^0	one minute

PCR was performed for 20 cycles using vent polymerase.

Following PCR, the product was electrophoresed through a 1.0% agarose gel and the HA1-K DNA was extracted from the gel using a Qiagen kit according to the manufacturer's instructions. The purified HA-K DNA fragment was digested with EcoRI and Kpn I and the fragment was cloned into pUC19 that had been digested with EcoRI and KpnI. The plasmid was used to transform E.coli AG-1 to amp^r. Individual colonies were picked and grown in LB-amp. The identity of plasmids with the correct insert was determined by restriction mapping. The resulting plasmid termed pUC19 HA-K was purified using a Qiagen kit according to the manufacturer's instructions.

The GM-CSF fragment was cloned by PCR.

Upstream Primer

5' ACGGTACCGCACCCACCCGCTCACCCATC

Downstream Primer

5' TAGGATCCCGGCCGTCATTTTTGGACTGGTTTTTTGCACG

The PCR primers generate a GM-CSF fragment with

1. 5' KpnI site that allows in frame translation from the $(G_4S)_2$ portion of the HA-K molecule to the start of the mature GM-CSF molecule at amino acid 18.

2. A termination codon at the 3' end of the GM-CSF

3. 3' BamHI site

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute
	Extension	72^0	one minute

PCR was performed for 20 cycles using vent polymerase.

Following PCR, the product was electrophoresed through a 1.0% agarose gel and the GM-CSF gene was extracted from the gel using a Qiagen kit according to the manufacturer's instructions. The purified fragment was digested with Kpn I and BamHI and the fragment was ligated into pUC19 HA-K plasmid that had been digested with KpnI and BamHI.. The plasmid was used to transform E.coli AG-1 to amp^r. Individual colonies were picked and grown in LB-amp. The identity of plasmids with the correct insert was determined by restriction mapping. The resulting plasmid termed pUC19 HA-K-GM-CSF was purified using a Qiagen kit according to the manufacturer's instructions.

Cloning of HA-K-GM-CSF into Yeast Expression Vector

PCR of pUC19 HA-K-GM-CSF was used to generate a DNA fragment encoding HA-K-GM-CSF for cloning into a yeast expression vector. The PCR product contains Eag I cloning sites for in-frame insertion into the yeast expression vector.

Upstream Primer

5' CTGAATTCCGGCCGGACACAATATGTATAGGC

Downstream Primer

5' TAGGATCCCGGCCGTCATTTTTGGACTGGTTTTTTGCACG

Conditions for PCR were:	Denaturation	90^0	one minute
	Annealing	60^0	one minute
	Extension	72^0	one minute

PCR was performed for 20 cycles using vent polymerase.

Following PCR, the product was electrophoresed through a 1.0% agarose gel and the HA-K-GM-CSF gene was extracted from the gel using a Qiagen kit according to the manufacturers instructions. The purified DNA fragment was digested with Eag I and ligated to the yeast expression vector ITK, that had been digested with Eag I. The ITK vector is designed for (1) replication in E.coli and (2) expression of genes in the yeast Saccharomyces cerevisiae after stable integration using homologous recombination. The vector contains:

1. Sequences for replication of the plasmid in E.coli
2. Yeast Gal promoter for expression of heterologous genes in media containing galactose.
3. PrePro – Synthetic DNA sequence, optimized for secretion and signal sequence cleavage of distal genes in yeast.
4. Unique Eag I site for cloning genes to be expressed.
5. Alpha terminator- DNA sequence for efficient termination of proximal genes.
6. Delta sequence that allows for stable integration of the plasmid by recombination with endogenous delta sequences in the yeast chromosome.
7. Antibiotic resistance gene allowing for selection in E.coli with kanamycin and selection in yeast with G418.

This plasmid was used to transform E.coli strain AG-1. Transformants were selected by growth on LB plates containing 100 ug/ml kanamycin. Individual colonies were grown in LB media containing kanamycin and plasmids were purified. Restriction digests determined orientation of inserts. The resulting plasmid ITK HA-K-GM-CSF was purified using a kit purchased from Qiagen according to the manufacturer's instructions.

The purified plasmid was linearized with Mfe 1 and used to transform the yeast strain Saccharomyces cerevisiae WDHY131 using lithium acetate (LiAc). A 10 ml culture of S.cerevisiae grown to saturation at 30^0C in YPD media (per liter/ 20g Bactotryptone; 20 g dextrose; 10g yeast extract) was used to inoculate 100 ml of YPD. The culture was grown at 30^0Cwith shaking for 3 hours. The yeast were harvested by centrifugation at 11,000 x g for 2

minutes and resuspended in 25 ml of sterile water. The yeast were centrifuged as above and resuspended in 1.0 ml of 100mM lithium acetate and transferred to a 1.5 ml microfuge tube. The yeast were pelleted by centrifugation at 12,000 x g for 15 seconds and the supernatant removed. The cells were resuspended in 0.5 ml of 100mM LiAc. 50 uL of cell suspension was added to individual microfuge tubes and centrifuged as above. Supernatant was removed. Transformation mix added to the yeast pellet consisted of : 240 uL PEG (50% w/v); 36 uL 1.0 M LiAc; 5 uL single stranded DNA (10mg/ml) and 1 ug of linearized ITK HA-K-GM-CSF in 75 uL of water. The mixture was vortexed to resuspend the cell pellet and incubated at 30^0 for 30 minutes. The cells were then shocked at 42^0C for 15 minutes, centrifuged to pellet cells and resuspended in 0.5 ml of YPD. Yeast were incubated in YPD media for 3 hours and plated on YPD plates containing 2 mg/ml G418. Plates were grown at 30^0C for 3 days until individual colonies appeared. To screen for expression of HA-K-GM-CSF, individual colonies were grown in 1 ml of YPD media at 30^0C for 2 days. The cells were centrifuged at 8,000 x g for 2 minutes and the YPD media removed and replaced with 1 ml of YPG media (per liter/ 20g Bactotryptone; 20g galactose; 10g yeast extract) for induction from the gal promoter. Yeast were grown in YPG media for 2 days. At this time, an aliquot was removed and cells were pelleted. The supernatant was tested for GM-CSF expression using an ELISA kit purchased from Endogen. The protocol was according to the manufacturer.

A colony expressing high levels of the chimeric protein was identified. Based on standard curve of soluble GMCSF, expression level is approximately 2.0 mg/L of soluble material. There is no decrease in expression levels in the absence of G418.

Scale Up Purification of GM-CSF-K-HA

For scaled-up purification of the chimeric protein, yeast were inoculated into 500 ml of YPD and grown for three days at 30^0C. Cells were pelleted by centrifugation at 12,000 x g for 2 minutes and transferred to an equal volume of YPG for an additional three days of growth. The cells were then pelleted by centrifugation at 12,000 x g for 2 minutes and the supernatant collected. The soluble material was applied to an immunoaffinity column of anti-murine GMCSF monoclonal antibody (Endogen) linked to cyanogen bromide-activated Sepharose 4B (Sigma). Coupling of the monoclonal to the Sepharose was performed according to the

manufacturer. Efficiency of coupling was monitored using OD_{280} and of binding of GMCSF to immobolized antibody was tested using soluble, commercially available material.

Soluble yeast-derived material was applied to the column and allowed to flow by gravity. The column was washed with: (a) 20 volumes of 0.15M NaCl, 25 mM Tris pH 7.4 (TN) (b) 5 volumes of 50mM Tris pH 8.0 (c) 20 volumes TN. Bound material was then eluted with 10 volumes of 0.15M NaCl, 25mM Tris pH 2.5. Eluted material was neutralized with 1/200 volume of 1.5M Tris pH8.8. The purified material was concentrated using a Microsep 3K centrifugal devise (Pall Gelman Laboratory). Yields of chimeric protein were determined by ELISA (Endogen) according to the manufacturer's instructions.

Purified GM-CSF-K-HA

Purified GM-CSF-K-HA was analyzed by western blot. Approximately 1ug of GM-K-HA per lane was electrophoresed along with soluble GMCSF. For western blot, gels were transferred to Protran BA83 (Schleicher and Schuell), blocked with TBS (Tris Buffered Saline) containing 0.05% Tween 20 and 2% Nonfat Dry Milk. The blot was incubated with primary antibody (rat monoclonal anti-murine GMCSF, Endogen) at 1:5000 dilution in blocking buffer for 2 hours at room temperature. The blot was washed with TBS-0.05% Tween 20. Secondary antibody, alkaline phosphatase conjugated anti-rat IgG (Sigma) was incubated at 1:10,000 for 1 hour at room temperature.

Decoration of Cells with GM-K-HA

Purified GM-CSF-K-HA was used to decorate CMS 5 murine fibrosarcoma cells. CMS 5 cells were grown in DMEM, 10%FBS, Penicillin-streptomycin, harvested by trypsinization and washed 3 times with RPMI 1640 (Gibco). Cells were diluted to 1x 10^6/ml in RPMI 1640 and 0.9 ml were aliquoted to siliconized tubes. Cells were incubated for 2 hours at 37^0C with shaking and then washed 3 times with PBS containing 2% FBS. Primary antibody, rat anti-murine GMCSF monoclonal, was incubated for one hour at 4^0C. Cells were washed as above, treated with FITC labeled anti-rat antibody (Sigma), and incubated for one hour at 4^0C. After additional washing, the cells were analyzed by flow cytometry, which confirmed the presence of GM-CSF-K-HA on the surface of the tumor cells.

Quantitation of GM-CSF-K-HA on the Cell Surface of CMS 5 Cells After Decoration

CMS 5 cells were harvested and washed as described above. 1 x 10^6 cells in 1 ml of RPMI 1640 were incubated with 1 ug of purified GM-K-HA. After incubation for 15 min, 30 min, 1 hour, or 2 hours at 4^0C, room temperature, or 37^0C, the cells were washed 3 times with PBS containing 2% FBS. The cell pellet was lysed with 50 microliters of PBS containing 0.15% deoxycholate and the detergent subsequently diluted by the addition of 200 microliters of PBS. The material was serially diluted with PBS and tested by ELISA (Endogen). Based on the amount of GM-CSF detected in the cell lysates, it was possible to quantitate the average number of GM-CSF molecules associated with each cell. For example, after a 15 min incubation at 4^0C, 58,700 molecules were present per cell. After a 15 min incubation at room temperature, 25,700 molecules were present per cell. After a 15 min incubation at 37^0C, 17,200 molecules were present per cell.

Effective Immunization with Tumor Cells Admixed with GM-CSF/Hemagglutinin Fusion Polypeptides

CMS-5 murine fibrosarcoma cells were grown to 70% confluence in DMEM, 10%FBS, Penicillin-streptomycin, harvested by trypsinization, and washed 3 times with RPMI 1640. Viability was determined by trypan blue staining of an aliquot and the cells were then resuspended at a concentration of 4 x 10^6 cells/ml and 1 ml aliquots dispensed into siliconized microfuge tubes. The cells were incubated with 1 ug (microgram) murine GM-CSF-K-HA or 10 ng (nanograms) HA-K-murine GM-CSF per 10^6 cells for 3 hours at 37^0 C. Cells were then washed 3 times with RPMI 1640 and resuspended at 4 x 10^6 cells/ml in RPMI 1640. An aliquot of the cells was removed and the amount of cell-associated GM-CSF measured by ELISA as described above. There were approximately 20,240 and 18,000 molecules/cell in the GM-CSF-K-HA and HA-K-GM-CSF groups, respectively. Cells for a control vaccine, to be administered without a molecule of the invention (or any other immunomodulator), were prepared in parallel.

The cells were irradiated at 3500 rads from a ^{137}Cs source. 8 week-old female Balb/c mice (which are syngeneic for CMS-5) were anesthetized by metofane inhalation and vaccinated subcutaneously in the left inguinal fold with 1 x 10^6 cells in 0.25 ml. Each mouse received cells from only one vaccine type. Seven days later, wild-type CMS-5 cells at 70% confluence were harvested and washed 3 times in HBSS. Viability was determined by trypan blue staining of an aliquot and cells were adjusted to 4 x 10^6/ml in HBSS. The previously vaccinated mice were

then injected subcutaneously behind the neck, under metofane anesthesia, with 2x 10^6 live, wild-type CMS-5 cells in 0.5 ml HBSS. The groups receiving the HA-K-GM-CSF and control vaccines each consisted of 5 mice, whereas the group receiving the GM-CSF-K-HA vaccine consisted of 4 mice because 1 mouse failed to awaken from anesthesia.

Tumor development was assessed daily by palpation and visual inspection. The observer was blinded to the vaccine received by each set of mice to ensure against bias. Mice were sacrificed by CO2 asphyxiation when tumors become unwieldy. All mice that had received the control vaccine developed tumors within 18 days after challenge with live tumor cells. In contrast, 100% of mice that had received the GM-CSF-K-HA vaccine and 60% of mice that had received the HA-K-GM-CSF vaccine remained tumor- free at the end of the experiment, 40 days after challenge. Thus, immunization with a composition comprising tumor cells and a molecule of the invention confers significantly longer tumor-free survival than immunization with a composition comprising tumor cells but not comprising a molecule of the invention.

Reduction of metastases in mouse models

B16F10 murine melanoma cells were harvested and washed three times in PBS. Cells were then suspended at 5 x 10^5 viable cells/ml in PBS, with viability determined by staining an aliquot of cells with Trypan blue. 100 ul of this suspension was injected into the tail veins of 8-10 week old female C57BL/6 mice. On day 1 or day 3 after tumor challenge, mice were immunized with 1 x 10^6 irradiated B16F10 cells subcutaneously in the left inguinal fold. Groups (3 mice each) received either cells alone, cells mixed with 1 ug soluble recombinant murine GM-CSF (Serologicals Corp.), or cells mixed with 1 ug of exogenous engineered cytokine of the invention comprising murine GM-CSF at the N terminus, a $(Gly_4Ser)_2$ flexible linker, and the HA1 domain of influenza A/PR/8/34 hemagglutinin at the C terminus. The latter composition comprised cell-bound exogenous engineered cytokine, as demonstrated by ELISA on washed cells.

Mice were sacrificed on day 12, the thoracic cavity opened with dissecting scissors, and the lungs removed *en bloc* by tracheal transection. Metastases were enumerated with a hand lens. In the mice immunized 1 day after challenge, the average number of metastases/mouse was as follows:

Cells alone: 30.00

Cells + GM-CSF: 14.33

Cells + exogenous engineered cytokine: 0.67

In the mice immunized 3 days after challenge, the average number of metastases/mouse was as follows:

Cells alone: 36.33

Cells + GM-CSF: 10.33

Cells + exogenous engineered cytokine: 1.00

Thus, administration of the composition comprising exogenous engineered cytokine an exogenous engineered cytokine of the invention was able to effectively reduce metastases and treat disease. The portion of the experiment in which mice were vaccinated on day 3 was repeated. In this instance, the average numbers of metastases/mouse were as follows:

Cells alone: 14.67

Cells + GM-CSF: 36.33

Cells + exogenous engineered cytokine: 5.66

In this experiment, spleens were also collected from mice at sacrifice to assay for tumor-specific, interferon gamma secreting T cells by ELISPOT. Spleen lymphocytes were plated at $1x10^5$ cells/well into IFNγ ELISpot plates (R&D Systems, Minneapolis, MN) along with $8 x10^5$ naive spleen feeder cells. B16F10 target cells were lysed by four repetitive freeze thaws and added at a concentration of 1x105 cells/well. Plates were incubated at 37°C, 5% CO2 for 24 hours and developed according to the manufacturer's protocol. Briefly, the plate was washed four times with provided wash buffer and incubated at 4°C over night with a biotinylated polyclonal

antibody specific for mouse IFN-gamma. The next day, following an additional 4 washes, 100 ul of a streptavidin – alkaline phosphatase conjugate were added to each well and incubated at room temperature for 2 hours. After four washes BCIP/NTB chromogen (5-Bromo-4-Cloro-3' Indolylphosphate p-Toluidine Salt, Nitro Blue Tetrazolium Cloride) were added for 1 hour at room temperature. The plate was finally repeatedly washed with deionized water and dried. Plates were then analyzed electronically (MVS Pacific, LLC, Roseville, MN) using an ELISpot reader system (Carl Zeiss Vision, Germany).

The approximate frequencies of tumor-specific, interferon gamma secreting T cells/10^6 splenocytes were as follows:

Cells alone:	590
Cells + GM-CSF:	410
Cells + exogenous engineered cytokine:	1,780

Thus, administration of a composition comprising an exogenous engineered cytokine was highly effective at modulating an immune response to antigens comprised by the cells of the composition.

The experiment was repeated once again with vaccination on day 3. In this instance, additional groups were included in which mice receiving the vaccine comprising the exogenous engineered cytokine were depleted of either CD4+ or CD8+ T cells by repeated intraperitoneal administration of anti-CD4 or anti-CD8 monoclonal antibody. The average numbers of metastases/mouse were as follows:

Cells alone:	90
Cells + GM-CSF:	74
Cells + exogenous engineered cytokine:	28
Cells + exogenous engineered cytokine	

(CD4-depleted mice): 45

Cells + exogenous engineered cytokine

(CD4-depleted mice): 80

Thus, the full effect of the therapeutic vaccine comprising the exogenous engineered cytokine was dependent on the presence of CD4+ and CD8+ T cells, indicating that the therapeutic vaccine acted through modulation of an immune response.

A similar lung metastasis experiment was performed using the CT26 murine colon carcinoma model. CT26 cells were harvested and washed three times in PBS. Cells were then suspended at 5 x 10^5 viable cells/ml in PBS, with viability determined by staining an aliquot of cells with Trypan blue. 100 ul of this suspension was injected into the tail veins of 8-10 week old female BALB/c mice. On day 5 after tumor challenge, mice were immunized with 1 x 10^6 irradiated CT26 cells subcutaneously in the left inguinal fold. Groups (4 mice each) received either cells alone, cells mixed with 1 ug soluble recombinant murine GM-CSF (Serologicals Corp.), or cells mixed with 1 ug of an exogenous engineered cytokine of the invention comprising murine GM-CSF at the N terminus, a $(Gly_4Ser)_2$ flexible linker, and the HA1 domain of influenza A/PR/8/34 hemagglutinin at the C terminus. The latter composition comprised cell-bound exogenous engineered cytokine, as demonstrated by ELISA on washed cells.

Mice were sacrificed on day 12, the thoracic cavity opened with dissecting scissors, and the lungs removed *en bloc* by tracheal transection after instillation of India ink. The lungs were bleached in Feketes solution and tumor nodules were enumerated with a hand lens. The average number of metastases/mouse was as follows:

Cells alone: 37.00

Cells + GM-CSF: 34.75

Cells + exogenous engineered cytokine: 20.25

Again, the method of the invention markedly reduced the average number of lung tumors per mouse, even though it was practiced 5 days after the tumors were initially established.

GM-CSF-K-HA-Mediated Protection Against Tumor Challenge In Vivo

As an allogeneic tumor vaccine model, C57BL/6 mice (haplotype *b*) were immunized with C3H (haplotype *k*)-derived K1735 melanoma cells, followed by challenge with C57BL/6-derived B16F10 melanoma cells. K1735 cells were grown to 70% confluence in DMEM with 10% FBS and penicillin-streptomycin, harvested by trypsinization, and washed 3 times with RPMI 1640. Viability was determined by trypan blue staining of an aliquot and the cells were then resuspended at a concentration of 4 x 10^6 cells/ml for K1735. One ml aliquots were then dispensed into siliconized microfuge tubes. The cells were incubated with 1 ug GM-CSF-K-HA (a fusion polypeptide consisting of murine GM-CSF at the N terminus, a $(Gly_4Ser)_2$ linker, and the HA1 domain of influenza A/PR/8/34) per 10^6 cells for 2 hours at 4^0 C. An aliquot of the cells was removed for measurement of cell-associated GM-CSF by ELISA. Mean cell-associated GM-CSF across two experiments was approximately 60,000. Cells that were not admixed with any polypeptide and, in one experiment, cells mixed with 1 ug soluble murine GM-CSF (Serologicals Corp.) were prepared in parallel as control vaccines.

The cells were irradiated at 3500 rads from a ^{137}Cs source. 8 week-old female C57BL/6 mice were anesthetized by metofane inhalation and vaccinated subcutaneously in the left inguinal fold with or 1 x 10^6 cells in 0.25 ml RPMI, along with a total of 1 ug GM-CSF-K-HA (including bound and free fusion polypeptide). Each mouse received cells from only one vaccine type. Seven days later, B16F10 cells at 70% confluence were harvested and washed 3 times in HBSS. Viability was determined by trypan blue staining of an aliquot and cells were adjusted in HBSS to 1 x 10^5/ml. The previously vaccinated mice were then challenged subcutaneously behind the neck, under metofane anesthesia, with 0.5 ml of the B16F10 cell suspension.

Tumor development was assessed daily by palpation and visual inspection. "Onset" was defined as the first day on which a tumor mass was both palpable and visible. The observer was blinded to the vaccine received by each set of mice to ensure against bias. Mice were sacrificed by CO2 asphyxiation when tumors become unwieldy. Experiments were terminated 70 days after tumor challenge, as planned in advance.

In pooled results from two experiments, 70 days after challenge, 7/10 mice that had been vaccinated with cells admixed with fusion polypeptide remained tumor-free. In contrast, 10/10 mice that had been vaccinated with cells alone developed tumors, as did 4/5 mice vaccinated with cells admixed with soluble murine GM-CSF.

4. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

March 24, 2004
Date

Andrew Segal